National Institute of Standards & Technology

Certificate of Analysis

Standard Reference Material 916a

Bilirubin

Standard Reference Material (SRM) 916a consists of a sample of bilirubin which is certified as a chemical of known purity. It is intended primarily for use in the calibration and standardization of procedures used for the determination of bilirubin in clinical samples and for routine evaluations of daily working standards used in these procedures. The certified purity of this SRM is:

$$98.3 \pm 0.3\%$$

The purity and estimated uncertainty is based upon scientific judgment and evaluation of the numerous analytical tests applied to this SRM in the certification process. The uncertainty is meant to approximate two standard deviation limits for the certified value.

The bilirubin used for this SRM was supplied by Pfanstiehl Laboratories, Inc., Waukegan, IL. It was prepared from material that was isolated from hog bile and crystallized as the acid. It was purified further by treatment in chloroform with sodium sulfate according to Fog [1] and recrystallization from chloroform.

Analyses for the certification and characterization of SRM 916a were performed in the NIST Center for Analytical Chemistry by R.G. Christensen, A. Cohen, B. Coxon, and M.J. Welch of the Organic Analytical Research Division and D.A. Becker of the Inorganic Analytical Research Division.

The overall direction and coordination of the technical measurements leading to the certification were provided by A. Cohen and E. White V of the Organic Analytical Research Division, Center for Analytical Chemistry.

Statistical consultation was provided by R.C. Paule, NIST National Measurement Laboratory.

The technical and support aspect concerning the preparation, certification and issuance of this Standard Reference Material were coordinated through the Office of Standard Reference Materials by R.L. McKenzie.

Gaithersburg, MD 20899
June 1, 1989

Stanley D. Rasberry, Chief
Office of Standard Reference Materials

(Over)

SUPPLEMENTAL INFORMATION: The results described below are provided for informational purposes only. These values are not certified but are provided because they may be of interest to the user of the SRM.

The bilirubin in SRM 916a consists of a mixture of three isomers of bilirubin [2]. The relative amounts of the isomers were determined by two methods. The mole fractions of the bilirubin IIIα, IXα, and XIIIα isomers measured by thin-layer chromatography (TLC), using 1% acetic acid in chloroform [2] on silica gel G were 5.3, 83.1, and 11.5%, respectively; and measured by high performance liquid chromatography (HPLC) on silica using approximately 1% acetic acid in dichloromethane were 7.5, 83.2, and 9.3%, respectively. The absorptivity of the material in chloroform (reagent grade containing 0.75% ethanol stabilizer) at 453 nm was 104.8 ± 0.2 $L \cdot cm^{-1} \cdot g^{-1}$ at 20 °C. A separately prepared solution, whose volume and absorbance were determined at 25 °C gave 105.0 ± 0.1 $L \cdot cm^{-1} \cdot g^{-1}$. Both values are uncorrected for the 1.2% chloroform, and other impurities in the crystals. The absorptivity reflects contributions from the three isomers of bilirubin present.

Samples of the SRM were dissolved in dimethyl sulfoxide-d_6 and measured by ^1H nuclear magnetic resonance (NMR). The amount of chloroform detected was 1.2%. Neither biliverdine, an oxidation product of bilirubin, nor mesobilirubin, the diethyl analogue, were detected by NMR. The chloroform present is tightly retained in the crystals. The loss of weight at 60 °C for 71 hours under vacuum was only 0.03%. Since the chloroform is so firmly retained, we recommend that the material be used as supplied. Mesobilirubin was not detected by mass spectrometry. Biliverdine was not detected by high performance liquid chromatography (0. 1% limit of detection) or by visible absorption spectrophotometry (0.01% limit of detection).

Impurities more acidic than bilirubin were determined quantitatively by extracting samples with 5% sodium bicarbonate, washing with chloroform, acidifying, extracting with chloroform, washing with water and concentrating chloroform extract. The dry weight of the extracts was 0.20%. Examination by thin-layer chromatography and by mass spectrometry after silylation indicated that some material similar to or identical to bilirubin had passed through the extraction procedure and was in the extract and thus the amount of impurity more acidic than bilirubin is less than 0.20%. Nonacidic impurities were 0.013%. They were determined by mixing a solution of 100 mg of bilirubin dissolved in chloroform with 0.1 *M* sodium carbonate. Bilirubin forms a sodium salt which is water soluble. This solution was extracted with chloroform, the extract washed with water, the chloroform removed by evaporation and the residue weighed.

An unidentified impurity was detected by thin-layer chromatography with polyamide as the adsorbent and 3:1 (v/v) methanol—aqueous ammonium hydroxide (3.3%) as the developer [3]. One hundred micrograms of SRM 916a bilirubin dissolved in chloroform gave a single yellow-orange spot at R_f 0.63, but under 365-nm radiation, a pink fluorescent spot, which was barely detectable, developed above the bilirubin spot at R_f 0.76. The quantity of material was estimated by comparing the intensity of the spot with spots of known amounts of bilirubin after spraying with a reagent consisting of phosphomolybdic-phosphotungstic acid (Folin-Denis reagent) and exposure to ammonia vapor. The amount of the unknown material was estimated to be 0.2%.

USE AND STORAGE OF THIS SRM:

This SRM is for "*in vitro*" diagnostic use only.

The SRM is supplied in a vial that has been capped under argon. The vial is sealed in a foil-lined polyester film bag also purged with argon. After opening, the material should be kept in the tightly-closed vial protected from light. Previous experience at NIST, where SRM 916 was stored at -15 °C and protected from light found the SRM to be stable for 3 years. SRM 916a stored under the same conditions should be equally stable. If the material degrades beyond the limits certified, purchasers will be notified by NIST. It is recommended that the material not be used after 3 years from the date of purchase.

This material is for use as a standard in clinical chemistry. An 0.2 $g \cdot L^{-1}$ standard solution for the preparation of a calibration curve may be obtained as follows [4,5]. Weigh out 20.3 mg (provides correction for purity) of SRM 916a bilirubin, transfer to a 100-mL volumetric flask, and dissolve by adding 1.0 mL of dimethyl sulfoxide and 2.0 mL of 0.1 *M* sodium carbonate. Dilute the solution to 100 mL with bovine serum albumin (fraction V, 40 $g \cdot L^{-1}$, previously adjusted to pH 7.4) or with an "acceptable serum diluent" for unconjugated bilirubin [6]. Since bilirubin is light sensitive, the flask should be immediately wrapped with aluminum foil. It is further recommended that the solution preparation be carried out with low-intensity incandescent light and absolutely away from any sunlight. Standard solutions of lower concentration may be prepared by dilution of appropriate aliquots with the serum albumin solution. It has been reported [7] that deterioration of the standard solution was about 1.5% per month at -20 °C, and about 1% in six months at -70 °C.

The molar absorptivity of SRM 916a Bilirubin in caffeine reagent [8], and of the blue and red azopigment products, obtained by the Reference Method for Total Bilirubin (developed by the Committee on Standards of the American Association for Clinical Chemistry) [9,10] were determined in a round-robin collaborative study. These values, shown in Table 1, are not certified, but are provided as useful information for the user of SRM 916a. The molar absorptivity value of the red azopigment at 530 nm was obtained by omitting the addition of alkaline tartrate in the Reference Method.

Table 1 Molar absorptivity

	Azopigments (Reference Method)		SRM 916a in Caffeine Reagent	
	λ = 598 nm	λ = 530 nm	λ = 457 nm	λ = 432 nm
Molar Absorptivity[a] ($L \cdot mol^{-1} \cdot cm^{-1}$)	76500[b]	56700[b]	49000[b]	50100[b]

[a]Corrected for purity of bilirubin.
[b]One standard deviation of the mean of the round-robin results is 300; one standard deviation of a single measurement is 700.

We gratefully acknowledge the contribution of the following, who participated in the round-robin study:

Hartford Hospital (Hartford, CT): R.B. McComb, A.O. Okorodudu
Beckman Instruments, Inc. (Brea, CA): W. Navazi, A. Kessner
St. Joseph Ziekenhuis (Eindhoven, Netherlands): H.L. Vader, K.L.J. Vink
National Institute of Public Health and Environmental Protection (Bilthoven, Netherlands): H.C. Koedam, G.M. Steentjes, R.H. Wikkeling, C.J. Phielix-Strubbe
Medical College of Wisconsin (Milwaukee, WI): B.T. Doumas, B.W. Perry, B. Jendrzejczak, L. Hubbard

REFERENCES:

1. Fog, J. Bilirubin-purification-purity. *Scand. J. Clin. Lab. Invest.* **16**: 49-54; 1964.
2. McDonagh, A.F., and Assisi, F. Commercial bilirubin: a trinity of isomers. *FEBS Lett.* **18**: 315-317; 1971.
3. Petryka, Z.J., and Watson, C.J. Separation of bile pigments by thin-layer chromatography. *J. Chromatogr.* **37**: 76-82; 1968.
4. Tietz, N.W. (ed.). Textbook of Clinical Chemistry, W.B. Saunders, Philadelphia, 1986, pp 1386-1393.
5. Perry, B.W., *et al.* A candidate reference method for determination of bilirubin in serum. Test for transferabily. *Clin. Chem.* **29**: 297-301; 1983.
6. Recommendation on a uniform bilirubin standard. *Clin. Chem.* **8**: 405-407; 1962.
7. Doumas, B.T., *et al.* Standardization in bilirubin assays: Evaluation of selected methods and stability of bilirubin solutions. *Clin. Chem.* **19**: 894-993; 1973.
8. Vink, K.L.J., et al. Use of the caffeine reagent in direct spectrophotometry of bilirubin. *Clin. Chem.* **32**: 1389-1393; 1986.
9. Doumas, B.T., et al. Candidate reference method for determination of total bilirubin in serum: Development and validation. *Clin. Chem.* **31**: 1779-1789; 1985.
10. National Reference System for Total Bilirubin, NCCLS Code NRSCL7-CR. Report on Analyte Reference Summaries of the National Reference System for the Clinical Laboratory (Revised 3/29/89, pp 20-21), National Committee for Clinical Laboratory Standards, Villanova, PA 19085.